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AN 134:233815 CA
TI Physical perturbation for fluorescent characteriz ation of microorganism particles
AU Bronk, Burt V.; Shoaibi, Azadeh; Nudelman, R aphael; Akinyemi, Agnes N.
SO Proceedings of SPIE-The International Societ for Optical Engineering (2000), 4036(Chemical and Biological Sensing), 169-180.

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AN 131:141627 CA
TI Fluorescence of dipicolinic acid as a possible component of the observed UV emission spectı a of bacterial spores
AU Nudelman, Raphael; Feay, Nicole; Hirsch, Mi thew; Efrima, Shlomo; Bronk, Burt
SO Proceedings of SPIE-The International Socie y for Optical Engineering (1999), 3533(Air Monitoring and Detection of C ıemical and Biological Agents), 190-195

3.
AN 135:192324 CA
TI Ultraviolet fluorescence imaging applications
AU Hill, Ralph H., Jr.; Angell, Peter
SO AT-PROCESS (2000), 5(3,4), 108-114

4
AN 123:51436 CA
TI Spectroscopic properties of tryptophan and b acteria
AU Tang, G. C.; Yang, Y. L.; Huang, Z. Z.; Hua W.; Zhou, F.; Cosloy, S.; Alfano, R. R.
SO Proceedings of SPIE-The International Soc ety for Optical Engineering (1995), 2387, 169-72

5.
AN 120:265052 CA
TI Online, non-destructive biomass determinat on of bacterial biofilms by fluorometry
AU Angell, Peter; Arrage, Andrew A.; Mittelma ı, Marc W.; White, David C.
SO Journal of Microbiological Methods (1993) 18(4), 317-27

Physical Perturbation for Fluorescent Characterization of Microorganism Particles

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ABSTRACT

The motivation for using response to physical perturbation to classify microparticles came from our previous experiments with Dipicolinic Acid (DPA). DPA as a calcium complex is a major component of bacterial spores, constituting more than 5% of their dry weight. It is not commonly found in other natural products and therefore its presence is indicative of the presence of bacterial spores. Previous schemes utilizing the presence of DPA to detect these spores have relied on fluorescence which occurs when lanthanide metals (e.g., terbium) are added to a solution where the presence of DPA is to be determined. We have recently demonstrated that changes in the fluorescence of DPA can be stimulated without the addition of such reagents Thus after exposure to UV light, a substantial increase of fluorescence emitted by DPA solutions with a peak at 410 nm occurs for excitation light with wavelength less than ~305 nm.

We have extended the above observation to obtain examples showing when this effect and comparable effects of other physical perturbations (temperature changes etc.) may provide very rapid information which can be used to initially classify particles. We have examined spores of Bacillus spp. and have found that large changes in the fluorescence of these particles and their supernatant comparable to that observed for chemical solutions occurs after short treatments with exposure to heat and UV light. Comparison of these results with those obtained with the same treatment of several other biological particles (e.g., vegetative cells of gram negative or gram positive bacteria etc.) shows that this effect of stimulated fluorescence indeed has the potential for preliminary and rapid classification of particles collected from an aerosol.

KEYWORDS

Fluorescence of Microorganisms; Optical Classification of Particles; Physical Perturbation of Microorganism Particles; Ultraviolet preirradiation for fluorescence

1. INTRODUCTION

In recent years, much progress has been made in developing instrumentation for rapid detection and identification of microorganisms particularly when the suspected particles arrive in the form of an aerosol. Instrumentation has been developed to detect autofluorescence with UV excitation[1-4] while the particles are still airborne. The fluorescent emission indicates when the presence or absence of biological particles in the aerosol and may be combined with light scattering data to give a measure of the size distribution of individual particles. The instrumentation is rapid, reliable, and can be designed to fit into a small package so it is fieldable. This instrumentation is quite suitable for incorporation into biodetection systems, although the

published report[1] does not indicate much in the way of discrimination between different types of microorganisms.

The most promising identification methods utilize biorecognition molecules, either antibodies, aptamers, or nucleic acid probes for DNA or RNA[5]. Each of these methods has advantages and disadvantages. They promise eventually to be both fairly rapid, and highly specific, but the best reported results at present using these methods show large variations for identification times with minimum times of ~30-60 minutes or more being the best reliably reported up to now[5]. The preparation time prior to the identification procedure may also be substantial. Quantitation of the concentration of pathogens present using these methods is also apt to have large statistical variation. In the event of the need for a rapid decision, such as determining when an area may be reentered following contamination, it would be desirable to have quantitative information available as rapidly as possible. Since the biorecognition method may have intrinsic physical limitations to its rapidity, and the autofluorescence method reveals very little about the identity of the biological particles, there is a niche available for instrumentation which is capable of very rapidly (e.g., less than five minutes) classifying types of microparticles in the environment using additional physical methods, even though it may not provide identification as certain as we expect will eventually be available using biorecognition molecules, nor be quite as rapid as the autofluorescence method for screening unknown aerosol particles.

In this presentation we introduce methods which combine a simple heating step with or without UV exposure of the particles together with spectral examination of the supernatant from the particles or of the particles themselves.. The experiments using these methods show promise, since the presence of bacterial spores is clearly indicated by resulting spectra, and there are indications of distinguishing features which would allow some additional rapid classification of the aerosol particles present. The procedures are cheap, automatable, potentially very fast, and require no additional reagents other than water and therefore we believe merit further exploration.

The work presented here was suggested by an earlier investigation into fluorescence obtained from dipicolinic acid (DPA, 2,6-pyridinedicarboxylic acid). DPA is a major component of bacterial spores comprising typically, 5 to 15% of the dry weight[6,7]. Detailed quantitative studies (e.g., ref. 4) of UV excited fluorescence from bacterial spores as well as vegetative bacteria showed that the main emission band has a smooth distribution between 300 and 400 nm with a peak near 330 nm. This emission has been attributed primarily to the amino acid tryptophan which has a similar band with a peak near 350 nm. It was thought however, that since DPA absorbs light in the region ~ 250 to 300 nm and it is present in large concentrations in bacterial spores, that DPA might be a contributor to spore fluorescence, particularly in the tail of the fluorescence curve well above 400 nm. We had earlier observed emission from DPA in the 400 to 420 nm region but this emission turned out to be hard to reproduce quantitatively[4]. We later discovered that the reason for this was that exposure of DPA to UV excitation in the fluorimeter and possibly even long exposure of DPA to fluorescent room lights affected the emission observed. After this was determined, it became straight-forward to obtain quantitatively reproducible results with measured exposure of DPA solutions to 254 nm light at controlled concentration and pH values[8].

2. Experimental

2.1 Instrumentation

Absorption measurements were made at room temperature with a Beckman Model DU 7400 diode array spectrophotometer with matched 1 cm path-length quartz cuvettes. Fluorescence measurements were made in 1 cm square quartz cuvettes with a Spex Fluorolog-2 spectrofluorometer equipped with double grating excitation and emission spectrometers. All slits of the monochrometers were open to 1 mm, which gave an excitation bandpass of 1.7 nm and a spectral emission resolution of about 3 nm. UV irradiations were carried out using the 254 nm band of a UVP Mineralight Lamp Model UVGL-58, with the use of a quartz cell at ~ 1 mW/cm^2 or 0.34 mW/cm^2 as indicated. The UV dosage was measured using a UVX digital radiometer with a 254 nm sensor. Heating of the spores or bacteria occurred in a Barnstead Thermolyne heater Model DB17615 in a 3 x 4 x 2 in deep aluminum heating block into which a 1 ml plastic microcentrifuge tube containing the spore or cell suspension was immersed. Various heating protocols were tried but a rapid immersion for 10 minutes at 97 C followed by quick chilling in ice and ten minutes centrifugation on a table

top centrifuge to separate supernatant from cells and debris was used for the results presented here with some minor departures which are noted.

2.2 Materials and Preparations

Dipicolinic acid (DPA, 2,6-pyridinedicarboxylic acid) and Ca $(NO_3)_2$ were obtained from Aldrich Chemical Co. DPA solutions were prepared freshly each day. The DPA stock solution was prepared each day by dissolving 5mM of DPA in 50 ml of twice the equivalent of NaOH with mild heating giving 0.1 M Na_2DPA. CaDPA was prepared from this by adding 1 part Na_2DPA stock to 4 parts 0.1M $Ca(NO_3)_2$. Phosphate Buffered Saline (PBS, pH 7.4) was reconstituted from a standard package obtained from Sigma. Microorganisms used in the experiments were *Escherichia coli* K 12 (ATCC 49539) *Bacillus subtilis* (strain Niger, termed BG) spores obtained as a dry powder from the U. S. Army Dugway Proving Ground stock. These spores were either cleaned by repeated centrifugation and resuspension in distilled water, or used directly from stock. Vegetative cells were grown from these spores or from cells obtained from slants. Growth was in LB liquid medium in a shaker bath with aeration . A third microorganism used was *Bacillus megaterium* (ATCC 13632) which is listed as a nonsporulating strain in the American Type Culture Collection catalog LB (ATCC medium 1065) consists of 10.0 g NaCl, 10.0 g tryptone. and 5.0 g yeast extract (Difco 0127) per liter of distilled H_2O)at pH ~ 6.9 —7.1.

3. Results

In Figure 1 we show the fluorescence of CaDPA excited at 300 nm. The CaDPA solution was exposed to 254 nm irradiation for various times as indicated, with a fluence of ~0.34 mW/cm^2. The solution was maintained at pH 7.5 with .01 M Hepes buffer. The buffer is not absolutely required, since the same results were produced in distilled water solution but were quantitatively erratic due to fluctuations in pH. We note that the emission curve is a smooth unimodal distribution with peak at 406 nm and a full width at half max of about 60 nm. It is slightly skewed to the right with a long wavelength tail extending beyond 550 nm. Similar results were achieved with Na_2DPA and DPA^{2-}. The shape of the emission spectra could be seen to be close to invariant for different excitation wavelengths between 270 and 310 nm which suggests that a single photoproduct is primarily responsible for the emission. If one plots the results for zero minutes of irradiation on an expanded vertical scale, one may see a similar graph with peak at ~ 406 nm. This could be due to a very small amount of the photoproduct, or a transient form of DPA complex which is always present in the solution.

The result shown in Figure 1 suggested that the appearance of a similar fluorescence emission graph after UV irradiation for unknown particles suspected of being bacterial spores could be a convenient method for confirming the presence of the spores. It turns out that this is the case. The effect is most pronounced when the DPA is extracted from the spores by the simple means of heating in water[10] as described in the experimental section but appears even without this step.

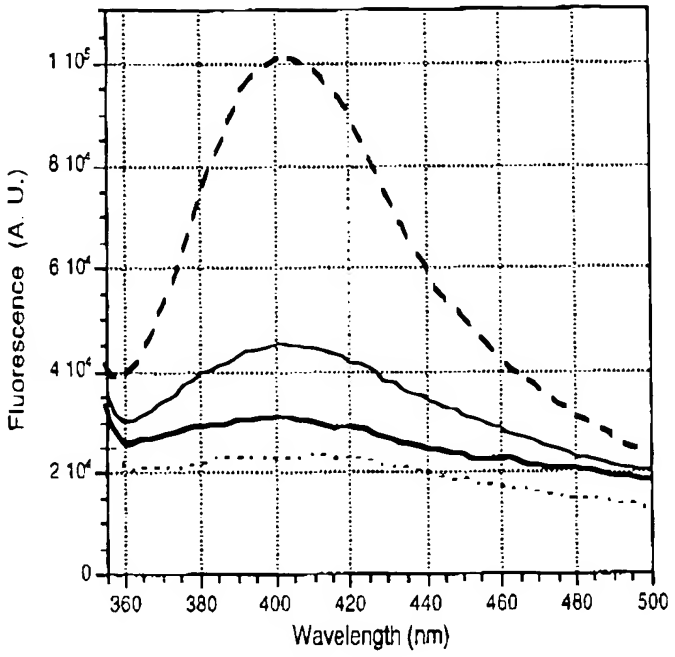

Figure 1. Fluorescence of 10 μM CaDPA solution in 10 mM HEPES hemisodium buffer (pH 7.5) after various times of 254 nm UV irradiation (fluence 0.34 mW/cm^2). 0 min, dotted line; 5 min, heavy solid line; 15 min, light solid line; 30 min, dashed line. Arbitrary units (A.U.) are used for fluorescence intensity in all figures.

In Figure 2, we show an absorption spectrum of CaDPA dissolved in water. This spectrum is well known[9] and has a distinct double peak with maxima at 271 and 278 nm and a shoulder at ~263 nm. The graph is normalized for comparison to the spectrum of the supernatant from BG spores shown in the same figure. The supernatant was prepared by heating ~ 10^8 spores/ml in water at ~ 90 C for 15 min and centrifuging out the spores and debris in a tabletop centrifuge. The spectrum from the bacterial spore supernatant retains the same location for both maxima as well as the dip between the maxima and the shoulder as are seen for the pure CaDPA so that we may conclude that CaDPA is a major component of the spore supernatant.

In Figure 3 we show the graphs for fluorescence for excitation at 305 nm of a sample of BG spores which has been washed numerous times, dried and resuspended in water ~ 10^7 spores/ml after various treatments. Excitation was at 305 nm. The peak at the left is due to the water Raman scattering at ~3600 cm^{-1}. Heating was at ~ 95 C for 10 min. The UV treatment was applied for various times as indicated in the Figure caption either before heating and centrifugation (i.e., in the spores) or to the supernatant after heating and removal of the spores by centrifugation. We see that in the present case, the fluorescence graphs are quite similar to that obtained from the pure CaDPA except that the peak is somewhat more pronounced in the case of no UV irradiation. The fluorescence increases with the time of irradiation and the graph is similar in each case. For UV treatment before heating the spores, the fluorescence still

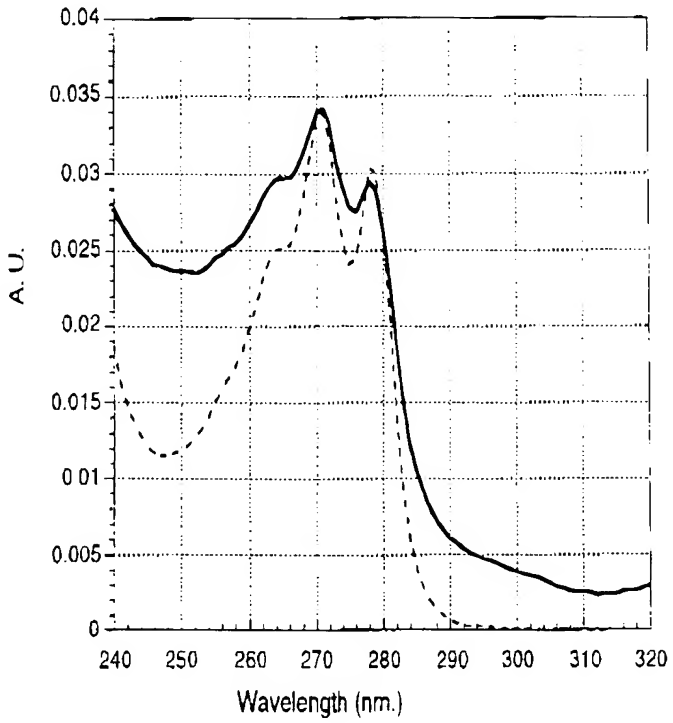

Figure2. Absorption spectra. CaDPA in water, dashed line; Supernatant from BG spores heated to 90 C for ~ 15 min, solid line. Arbitrary units (A.U.) are used and the spectra have been normalized to approximately same peak height.

increases substantially with UV treatment, but markedly less than for UV irradiation of the supernatant after heating the spores.

A similar fluorescence experiment was performed with BG spores which were not so highly processed with washings. These spores in dry bulk are red in appearance in contrast to the highly washed spores which were yellowish white. This is probably due to growth medium and growth products remaining on the surface of the spores. The spores were suspended in water at a concentration of ~ 3 x 10^7 spores/ml. The results of the experiment are shown in Figure 4 for excitation at 300 nm. The untreated spores in suspension show a small amount of fluorescence peaking at ~ 420 nm. The supernatant from the untreated spores shows this low fluorescence somewhat more sharply peaked. When the spores were subject to 10 min heating at ~ 97 C, and the supernatant from these spores was examined for fluorescence. the emission band became slightly more pronounced. When the supernatant taken after this heating was subjected to 10 min of UV irradiation at 0.34 mW/cm^2 the emission band becomes greatly enhanced with the peak still at ~420 nm. The shape of the peak is much more triangular and not as similar to that obtained from CaDPA as was the case for the highly processed spores indicating a complex spectrum with fluorescent chemicals in addition to CaDPA present.

Further, there is greater fluorescence at higher wavelengths. These changes may be due to material left from the growth medium and/or growth products.

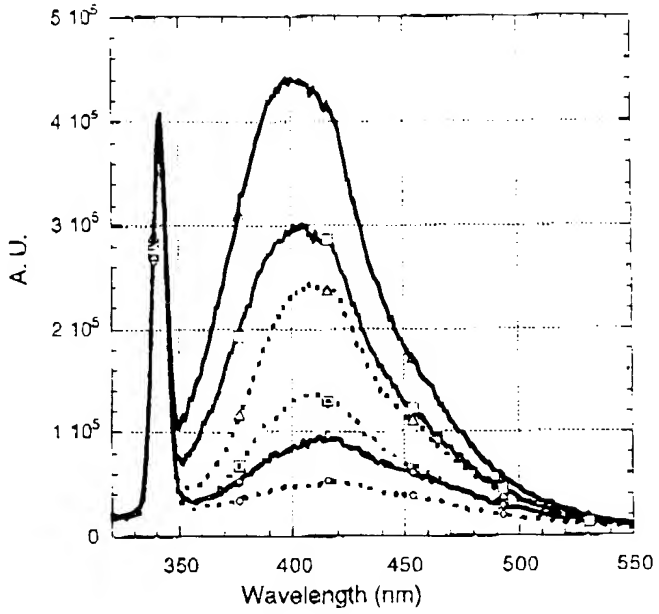

Figure3. Fluorescence with excitation of 305 nm from supernatant of highly washed BG spores after treatment by heat and UV in order indicated in text. UV radiation at 1 mW/cm^2 The duration of UV irradiation is denoted: 0 min (none) —circles; 10 min — squares; 30 min — triangles. Solid line with circles, heating and centrifugation only. Dotted line graphs — UV before heating and centrifugation (i.e., irradiation in the spores). Solid line graphs, UV irradiation of supernatant after heating and centrifugation.

To determine whether the fluorescent behavior after these simple treatments were performed could be used as a reliable indicator that bacterial spores were present, a number of other microorganism particles must be examined with the same experimental procedure. One such experiment is shown in Figure 5. This experiment is performed with a similar protocol on *Escherichia coli* K12 cells after overnight growth in LB medium with aeration. The cells were then washed and resuspended in PBS. In this experiment, UV irradiation is heavy (1 hr at 1.0 mW/cm^2) and the cells and supernatant were not seperated before the fluorescence spectrum was taken. The reason for this change is to exaggerate the effect of the irradiation on the fluorescence for illustrative purposes.

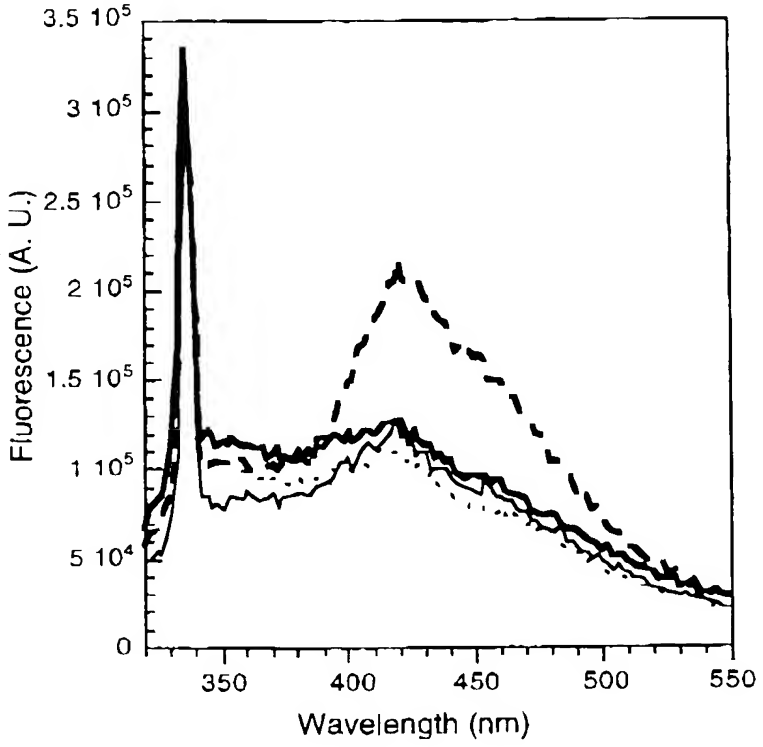

Figure 4. Fluorescence emission obtained from supernatant from unprocessed spores for excitation at 300 nm. Dotted graph is from spores in suspension with no heat or UV treatment. Thin solid line is for the supernatant from this suspension with no heat or UV treatment. Heavy solid line graph is an emission spectrum taken after this suspension was heated for 10 min at 97 C and the supernatant examined for emission after centrifugation but with no UV irradiation. The heavy dashed line is taken after the supernatant from the heated spores has been subjected to UV for 10 min at ~ 0.34 mW/cm^2.

Comparing the spectrum for 300 nm excitation from the UV irradiated cells with that obtained from the unirradiated cells, we see a much different effect than is obtained with the experiment with either washed or unprocessed BG spores. In this case, an emission peak not present for the spores appears at about 350 nm, but is substantially reduced by the UV irradiation. The emission graph for the irradiated case is elevated above the unirradiated case for wavelengths higher than ~400 nm.

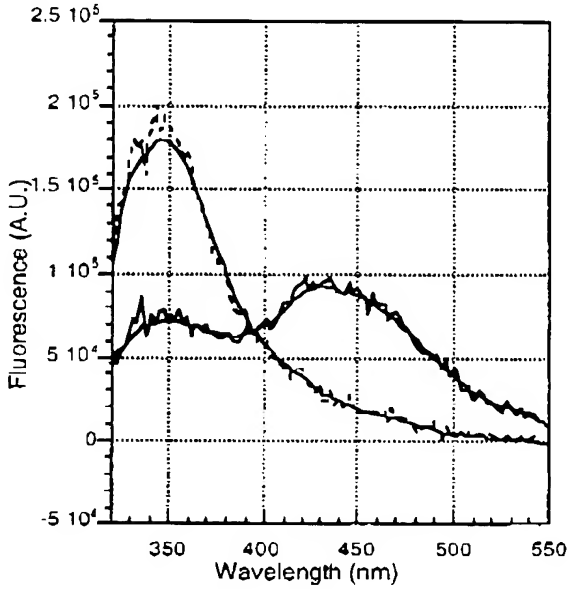

Figure 5. Graphs for fluorescence with 300 nm excitation of an overnight growth of *Escherichia coli* cells and supernatant after a similar treatment as was performed on BG spores for the graph of Figure 4. Dotted line, heat for 10 min, but no UV. Heavy solid line, same heating but with heavy dose of UV (see text). The Raman peak has been removed by subtracting background, and a smoothed curve drawn through the experimental points in both cases.

In Figure 6 we show a graph for the same treatment of the supernatant taken from an overnight growth of *E. coli* cells as was given to the BG spores in the cases shown in Figures 3 and 4. After overnight growth the cells were washed twice in PBS and then heated in PBS, then the supernatant was treated in various ways as indicated in the figure caption. The treatment was identical with that of the unprocessed spores shown in Figure 4, and the irradiation dose was six times as heavy. The reason the experiment with a heavier UV dose is shown is that the effect increases with dose, and is hardly visible for wavelengths above 400 nm with lower doses.

We see the same effect, but substantially smaller, than was seen for the experiment of Figure 5 where the supernatant together with the cells are irradiated or not both after heating and then the resulting suspension with supernatant and cells was excited by 300 nm light. The irradiated suspension in that earlier case shown in Figure 5 experienced three times the dose of the present case. Again, in Figure 6, the peak at ~ 350 nm is somewhat suppressed and a larger emission tail develops for the irradiated case both with and without heating

We see that the effect of the heating plus UV is substantially and recognizably different for Escherichia coli cells than it is for BG spores.

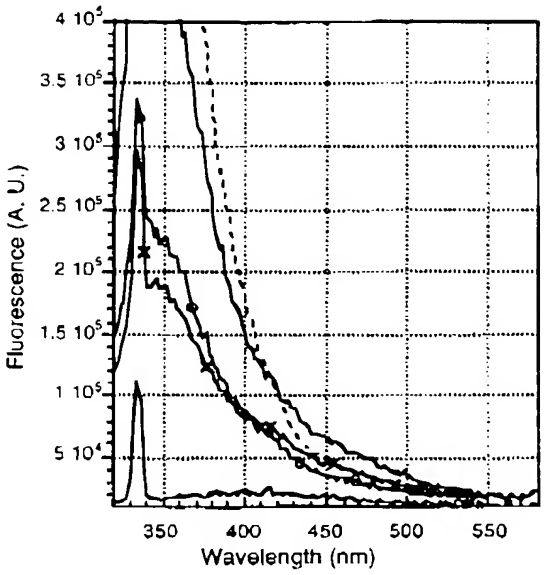

Figure 6. Fluorescence from supernatant after various treatments of *Escherichia coli* cells with 300 nm excitation. Heating was at 97 C for 10 min. UV irradiation was for 60 min. at ~ 0.34 mW/cm^2. The graph with the thin line at the bottom shows the background from PBS only. The thin solid line with circles is for the supernatant from unheated cells with no UV. The thin solid line marked with x s is for this supernatant from unheated cells with UV irradiation. The heavy dashed line is for supernatant from heated cells with no irradiation. The heavy solid line is for supernatant from heated cells with irradiation.

We also experimented with the present protocol using vegetative cells from either BG or from a non-spore forming strain of *Bacillus megaterium*. The results for BG vegetative cells in mid-log phase are shown in Figure 7. The cells were grown from spores overnight, diluted and regrown to midlog phase. They were centrifuged and resuspended in PBS, then washed once, then heated or not as noted in the Figure, then treated with UV or not for 10 minutes at 1.0 mW/cm^2. The results are quite distinguishable from those obtained from BG spores and are also distinguishable, although similar to the results obtained from *E. coli* cells with identical treatment.

The graph for the supernatant from *B meg.* cells with no heat and no UV is similar to that obtained from spores. The graph obtained for no heat but UV treated is somewhat similar to the results for the heat plus UV case for spores shown in Figure 4. The graphs for the cases with heating behave more similarly to the E. coli case of Figure 6, but not at all like either of the BG spore cases shown in Figures 3 or 4

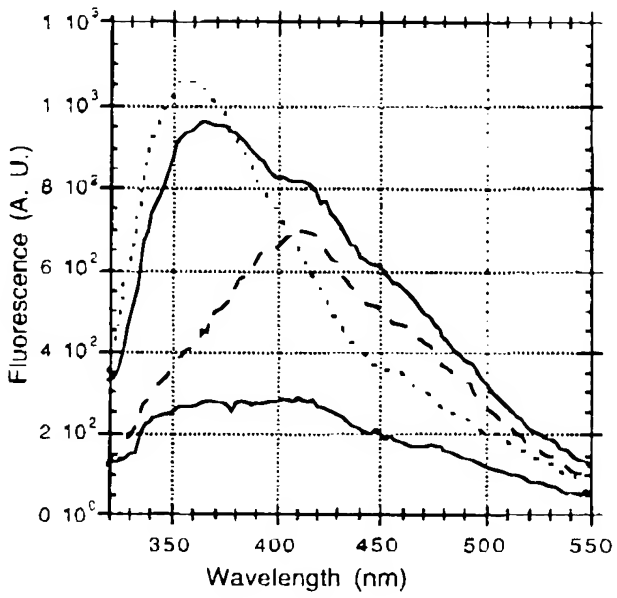

Figure 7. Fluorescence of supernatant after treatment for vegetative BG cells with 300 nm excitation. Thin solid line No Heat, No UV; dashed line No Heat plus UV; dotted line Heat, No UV; heavy solid line, Heat plus UV. Background subtracted and curves smoothed.

Additional fluorescence spectra were taken for all cases at other excitation wavelengths, with additional features revealed in some cases, but not shown in this presentation. For example, *Bacillus megaterium* vegetative cells showed fluorescence spectral patterns different from spores and also different from the other vegetative cells used in this presentation.

The absorbance spectra also reveal differences for the behavior of different microorganisms subjected to these treatments. In Figure 8, we see the absorbance spectra for *Escherichia coli* cells in suspension and supernatant, with or without heat treatment. In each case, the cells were resuspended in PBS after the supernatant was removed following centrifugation In all cases shown, UV was applied. (We found little if any effect of the UV doses applied here on absorbance). Heating reduced the absorbance maximum for the cells between 250 and 300 nm and increases the absorbance at a corresponding position for the supernatant. This suggests that the heat has caused a great deal of the absorbing material of the cells (including the fluorescing aromatic amino acids) to be released to the supernatant. We note that the pattern of the absorbance spectra for supernatant from these cells is quite different from that arising from the supernatant from bacterial spores which was illustrated in Figure 2.

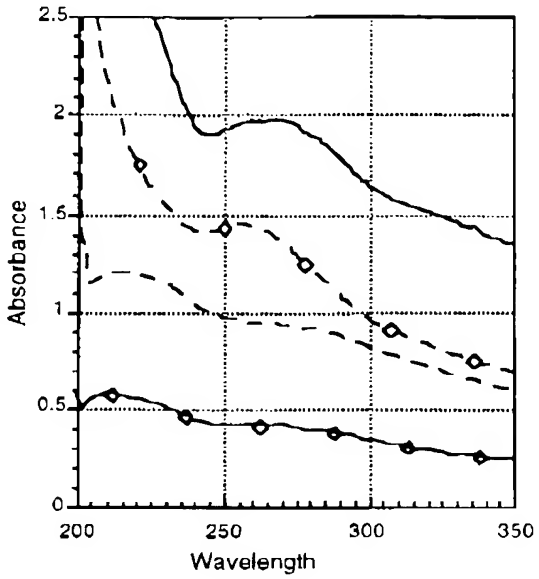

Figure 8. Absorbance spectra for *Escherichia coli* cells resuspended after supernatant is removed following treatment or for the supernatant. Solid line graphs represent no heat treatment; dashed lines represent with heat treatment. Graphs with diamond markers are for supernatant, the other two graphs for cells.

4. Conclusions

We have shown that with rather simple physical treatments that spores show fluorescence spectral patterns quite different from that observed for vegetative cells from the same or other species of microorganism. The experiments shown here cover a very limited range of treatments, bacterial species, and excitation wavelength. (We have extended the experiments to *Bacillus cereus* spores, and obtained similar results to those for the BG spore.) Even with these limitations, the results suggest that more detailed investigation will reveal patterns which can give some discrimination between bacterial species with simple automatable spectroscopic procedures where the treatment does not require any extensive use of reagents. The extent to which these procedures can be utilized for dirty samples and in the presence of interferrants remains to be determined. It is believed that other photonic interactions, perhaps with the use of two-photon events using higher wavelengths may lead to further photochemical interactions which can lead to some classification of microorganisms Finally, we mention that we have microscopically imaged spores and bacteria after procedures similar to those used in the present investigation with corresponding visible fluorescence indicating these results can be obtained from single microorganisms.

We acknowledge the collaboration of Dr. V. Sharma in recent experiments using *Bacillus cereus* spores.

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